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Platelet Aggregates as Markers of Platelet Activation: Characterization of Flow Cytometric Method Suitable for Clinical Applications

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The present paper describes a flow cytometric method for assay of platelet aggregates (PAg) in blood. This method combines and simplifies previously reported techniques, simultaneously enumerating PAg formed upon platelet activation, their expression of activation marker CD62P (P-selectin), and their content of bound leukocytes (LPAg). The sensitivity of this method to low levels of agonists (ADP, collagen) is compared to conventional aggregometry and some features of platelet-leukocyte interaction are explored. The results were: (1) ADP or collagen induced a dose-dependent increase in PAg number and corresponding decline in free platelets. The ED_{50} for ADP (0.15 μM) and for collagen (0.2 μg/mL) was about 1/20 the ED_{50} found by aggregometry, indicating 20-fold greater sensitivity. (2) At higher concentrations, the fraction of PAg with bound leukocytes (LPAg) increased to 60–70%. This rise correlated with PAg size and CD62P expression, but not with the number of PAg formed. (3) The response of whole blood (WBD) to agonists was qualitatively different from that of platelet-rich plasma (PRP): in WBD the population of CD62P+ PAg was much higher than in PRP and the population of CD62P+ free platelets was much lower. This implies that leukocytes rapidly recruit activated platelets. (4) Desmopressin (DDAVP) at 5 nmol/L induced a significant rise in activated (CD62P+) PAg and platelets, even though no effect of DDAVP could be detected by conventional aggregometry; this further confirms that DDAVP acts directly on platelets. (5) Plasma samples from TTP patients induced a rise in PAg when added to normal PRP, though little or no effect could be detected by aggregometry. In summary, the flow cytometric method described here appears useful for detecting low levels of platelet activation and provides information on platelet leukocyte interaction, potentially important in identifying and differentiating thrombogenic states. Since it is rapid and economical, it is well suited for clinical implementation. Am. J. Hematol. 57:33–42, 1998.

Key words: platelet activation; platele aggregates; platelet-leukocyte interaction

INTRODUCTION

Platelets play a central role in hemostasis and thrombosis, participating in many biologic and pathologic processes including inflammation, atherosclerosis, and immune reactions [1]. These activities require platelet activation, a complex process involving shape change, release of granular constitutants, adhesion, and aggregation [2,3]. Upon activation, platelets express adhesion molecules at their surface, initiating interaction with other cells such as endothelial cells and leukocytes. The extent and steps of platelet activation may differ in various disorders, but these difference have not been well described, in part because of limited available methodologies.

Among the many techniques introduced to assess platelet function, platelet aggregometry [4] is most widely accepted in the clinical laboratory. However, for certain purposes, platelet aggregometry is of limited sen-

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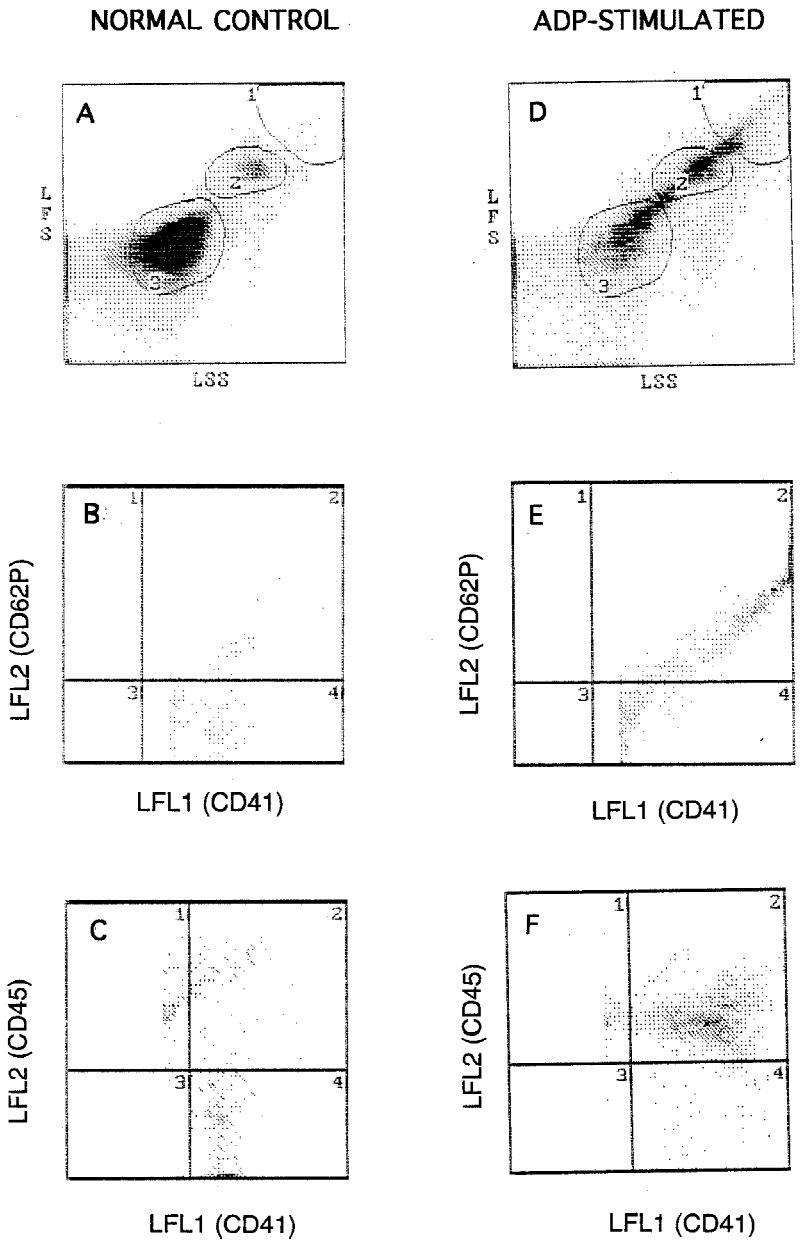

Fig. 1. Flow cytometric histograms of platelets and platelet aggregates (PAg). Whole blood was treated and analyzed as described in Methods. Histograms A,B,C were obtained with blood from a normal control and D,E,F were from the same normal control after stimulation with 0.5 µmol/L ADP. As shown in A, the CD41+ particles from the normal control were separated into three populations on the basis of forward-scattered light (LFS, y-axis) and side-scattered light (LSS, x-axis). Free platelets are represented by the dense population in bitmap 3, and platelet aggregates (PAg) are shown at the top right in bitmap 1. Calibration with standard beads showed the PAg to be >10 µm diameter. The intermediate population (bitmap 2) is referred to as ''micro-PAg'' and ranges from 5–10 µm diameter. Each axis scale spans 4 log decades. D shows the same sample but following stimulation with ADP. B and E are derived from bitmap 1 of A and D, respectively, and show fluorescent intensity of FITC on the x-axis, and of phycoerythrin (PE) on the y-axis. The FITC is the label on the platelet specific MAb, CD41, and the PE is the label on the activation marker, CD62p. Activated (CD62p+) PAg are defined as those having fluorescent intensity higher than that indicated by the horizontal line, i.e., in region 2 (top right rectangle). Notice the much greater number of CD62p+ particles in E (ADP stimulated). C and F are also derived from bitmap 1 of A and D, respectively, but here the PE label is on leukocytes (CD45) on the y-axis. Particles above the horizontal line (in region 2) are positive for CD45; co-expression of red and green (PE + FITC) indicates binding of PAg to WBC. Notice that F is much richer in CD45+ PAg than C.

sitivity and specificity. For example, the importance of platelet-leukocyte interactions in inflammation and thrombosis is now well appreciated [1,5,6] but aggregometry gives no information on this measure.

Flow cytometry has been applied to the detection of markers of platelet activation, such as CD62P (P-selectin), the presence platelet aggregates, and binding of platelets to leukocytes. In the present report, we have combined and simplified the methods of Ault et al. [7], Galt et al. [8], and Rinder et al. [9] to allow simultaneous measurement of all three measures: CD62P expression, platelet aggregates (PAg), and leukocyte-associated platelet aggregates (LPAg) in fresh samples of whole blood. These measurements complement the assay of platelet microparticles (PMP), another marker of platelet activation, whose clinical applications were reported earlier [10–14].

The present report documents the sensitivity of this method to various agonists in vitro, compares it to conventional aggregometry, investigates some events involved with the formation of PAg and LPAg, and explores its clinical utility in some patient groups where conventional aggregometry failed to detect abnormality. It is rapid and economical and is therefore well suited to the needs of clinical testing. This method, originally reported briefly in Jy et al. [15], has been applied to study the clinical courses of TTP patients and was found useful in monitoring effects of treatments and as a harbinger of exacerbation or remission [16].

METHODS

Materials

ADP and collagen were purchased from Sigma (St. Louis, MO). FITC-labeled α-CD41 and PE-labeled α-CD45 were purchased from Coulter Corp. (Miami, FL). PE-labeled α-CD62P was purchased from Becton-Dickenson Corp. (San Jose, CA). All of the monoclonal antibodies (MAb's) were used without dilution, as supplied by the manufacturers.

Sample Preparation

Blood (4.5 mL) was drawn from the antecubital vein of normal donors into Vacutainer tubes containing 0.5 mL of 3.8% sodium citrate. To 50 μL citrated whole blood in a 12 × 75 mm polypropylene tube was added 5 μL FITC α-CD41 to label platelets, and 5 μL PE-labeled α-CD62P to label activation marker P-selectin. In another tube, the α-CD62P was replaced with 5 μL PE α-CD45 to label WBC. After 10 min, samples were incubated with agonist (ADP, collagen, etc.) for 5 min with orbital shaking at 120 RPM, then fixed by adding p-formaldehyde (2% final), incubated for 10 min, and diluted with 1.0 mL of PBS. After 2 h, the samples were assayed by flow cytometry. The fixation procedure was found to be important: fixation time of >10 min led to inconsistent results and interference from unlysed RBC; if fixation was done before MAb addition, poor labeling (binding) resulted.

Flow Cytometry

Flow cytometry was done in the Coulter Profile II. The sample flow rate was 20 μL/min with sheath pressure of 4 psi, and each sample was measured for 60 sec; in view of 1:20 dilution factor, each measurement is equivalent to 1.0 μL whole blood. Laser output was 15 mW. Events were counted by triggering on a preset threshold of FITC fluorescence of platelet marker α-CD41 (GPIIb/IIIa). The threshold was set above the background fluorescence with FITC-labeled isotypic mouse MoAb. The CD41+ particle populations were separated by bitmaps (see histogram 1 in Fig. 1), where log forward scatter (LFS) is the y-axis and log side scatter (LSS) is the x-axis. Representative print-outs are shown in Figure 1. Three bitmapped populations are seen in Figure 1A and D. Bitmap 3 encloses the normal platelet population (<5 μm); bitmap 2 encloses micro-PAg (5–10 μm); and bitmap 3, at top right, defines PAg (>10 μm). Size was judged by calibration of forward scatter with standard beads of 5, 10, 15 μm (obtained from Polysciences Corp., Warrington, PA). The PAg population (bitmap 1) was then sent to its own histogram for two-color analysis, as seen in Figure 1B,E where the axes are CD41 and CD62P fluorescence, and Figure 1C,F where the axes are CD41 and CD45 fluorescence. This allows determination of the extent of CD62P expression or leukocyte bindings (CD45+) in PAg. Contamination of RBC in PAg population was found to be <2%.

The population in bitmap 2 (micro-PAg) is of uncertain significance and is seen in variable but low levels even in normal controls. This population may be an artifact reflecting a low level of platelet activation, possibly caused in drawing blood; because of this uncertainty we do not include measurement of this population in the analysis.

Estimation of Number of Platelets per PAg Particle

Two methods were employed: (1) By comparing the volume of a single platelet (~9 fL) to that of the PAg, assumed spherical, it was determined that the mean PAg (~10–14 μm diameter) consists of 60–160 platelets. (2)

By comparing the population of free platelets disappearing as PAg appear, it was found that ~100 platelets disappear for each PAg formed. These are only rough estimates because of complicating factors such as the presence of WBC in the aggregates, and the fact that forward-scattered light is only approximately representative of size.

Aggregometry

Aggregometry was performed in parallel on whole blood (WBD) and on PRP. The former was done in a Chronolog ''Whole Blood Lumi Aggregometer'' Model 500CA and the latter in a Chronolog Model 600 (Lancaster, PA), in accord with the manufacturer's instructions. The course of aggregation was followed for 5 min after addition of ADP or collagen. Stirrer speed was 1,000 RPM. To test the effects of TTP patient plasma on platelet aggregation, 500 μL of patient platelet-poor plasma (PPP) was added to 500 μL of normal PRP.

Statistical Analysis

Results in the graphs show mean and standard deviation of 4–6 experiments. Significance of differences between means was determined by the Student's *t*-test.

RESULTS

Analysis of Platelet Aggregates (PAg)

The appearance of whole blood from a normal volunteer as seen in flow cytometry is shown in Figure 1A–C. As described in Methods, histogram 1A shows separation of the populations in bitmaps 1–3 by side-scattered (LSS) and forward-scattered light (LFS). All particles are CD41+ (FITC label). The PAg of bitmap 1 (top right in Fig. 1A) are sent to histogram 1B for 2-color analysis: FITC fluorescence of CD41 is the x-axis (LFL1) and PE fluorescence of CD62P is the y-axis (LFL2). In Figure 1C, the CD62P label has been replaced with the CD45 label to evaluate binding of leukocytes to PAg.

Figure 1D–F shows the same sample after activation by sub-maximal ADP. Notice in Figure 1D the depletion of the platelet population (bitmap 3) and enrichment of the micro-PAg (bitmap 2) and PAg (bitmap 1). Figure 1E shows that both the number and percentage of PAg that were CD62P+ (region 2) are increased relative to Figure 1B. Figure 1F shows a similar increase in association of PAg with WBC; i.e., co-expression of FITC from CD41 (platelets) and PE from CD45 (leukocytes).

Sensitivity of Flow Cytometry Compared to Aggregometry

We compared the sensitivities of flow cytometry and platelet aggregometry in detecting platelet aggregates stimulated by ADP or collagen based on the ED50 obtained from their dose-response curves. ED_{50} is defined as the concentration of agonist required to induce 50% of maximal aggregation in aggregometry or maximal number of PAg formed in flow cytometry. As shown in Figure 2A,B, with increasing concentrations of ADP or collagen, the number of activated (CD62P+) PAg rises regularly. The ED_{50} of ADP and collagen-induced CD62P+ PAg formation measured by flow cytometry was 0.15 μmol/L and 0.2 μg/mL, respectively (Fig. 2A, B). In contrast, the ED_{50} of ADP and collagen-induced aggregation measured by conventional aggregometry on PRP was around 3 μmol/L and 4 μg/mL, respectively; and the ED_{50} of these reagents when used with the whole blood aggregometer was about 10 μmol/L and 15 μg/mL, respectively. Figure 2A,B shows that flow cytometry is about 20-fold more sensitive than aggregometry in detecting the effect of ADP or collagen on PRP, and about 50-fold more sensitive than whole-blood aggregometry. The greater sensitivity of the flow cytometric method at low levels of agonist is probably attributable to its capability of detecting very small platelet aggregates (''incipient aggregation'') whereas the aggregometer detects only macroscopic aggregates. However, at higher concentrations of agonist (>1.0 μmol/L of ADP or >1.0 μg/mL of collagen), flow cytometry fails to detect any further increase in the number of CD62P+ PAg, although their size continues to increase and their composition shifts, as described below.

It is seen in Figure 2C,D that the number of activated free platelets rises only modestly with increasing ADP or collagen. We interpret this to mean that as platelets become activated they readily interact to form PAg.

Characterization of PAg

The size and composition of the PAg was also studied, with particular attention to leukocytes bound to the PAg.

Size of aggregates. Prior to stimulation with the agonist, PAg are rare in normal plasma. As shown in Figure 3, after stimulation with the agonist the mean size of the PAg increased with increasing ADP. This was in contrast to the number of PAg, which plateaued at ADP >0.5 μmol/L (Fig. 2). These data suggest that at higher concentrations of agonist, recruitment of additional platelets to existing aggregates results in macroscopic aggregation.

Assessment of WBC in PAg. To assess the presence of WBC bound to platelets or PAg, duplicate tubes were prepared in which the α-CD62P was replaced with the pan-leukocyte marker α-CD45. As shown in Figure 1C,F, the PAg population may be broken down according to combinations of markers of CD41 and CD45: region 4 contains PAg having fluorescence only from FITC

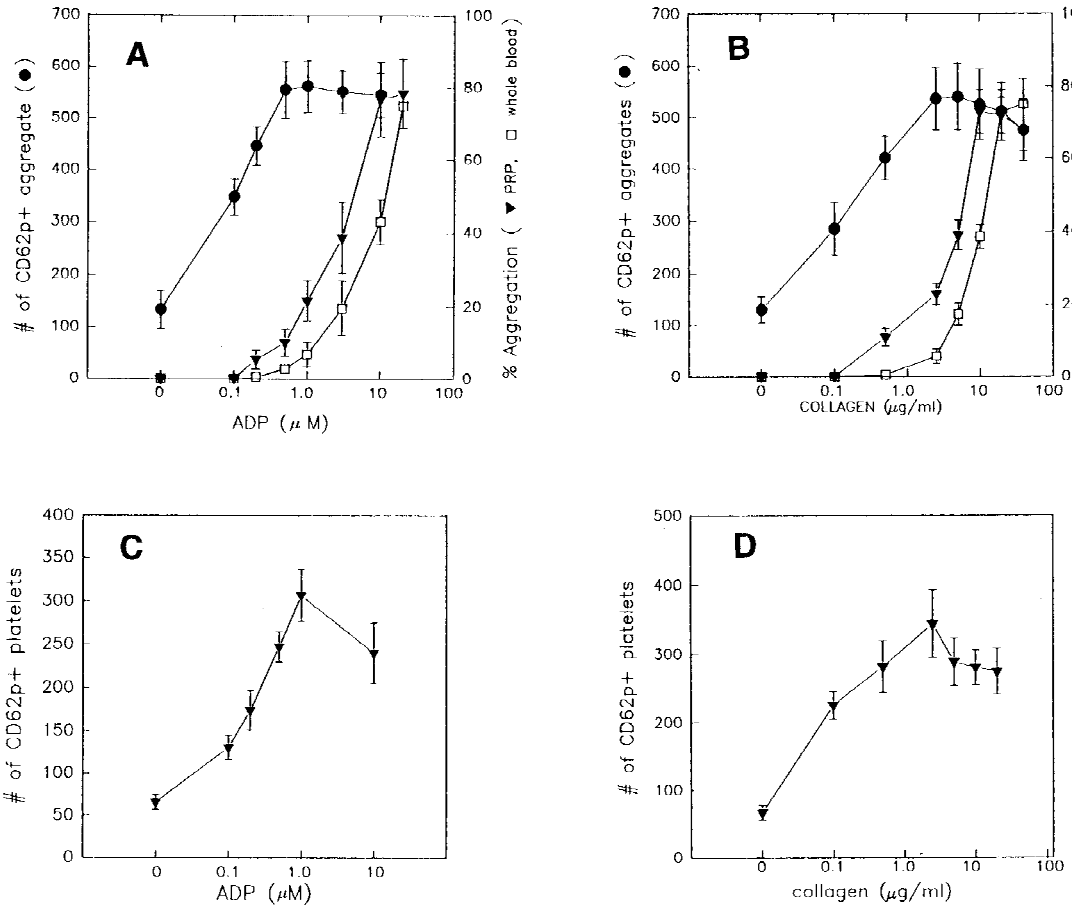

Fig. 2. A,B: Comparison of flow cytometry with agregometry of PRP (transmittance based) and whole blood (impedence based) in detecting agonist-induced platelet activation. Stimulation was by ADP or collagen, as indicated. The dose-response curve by flow cytometry is indicated by the solid circles, and units are expressed as absolute number of PAg formed per µL of whole blood. The response curve forPRP is indicated by solid triangles and units are expressed as a percentage of maximum aggregation, defined as the transmittance of platelet-poor plasma (PPP). Error bars are 1 S.D. The response curve for whole-blood aggregeometry is indicated by open squares; scale unites are as for PRP. C,D: Activated (CD62p+) free platelets as a function of agonist concentration. Units are expressed as absolute number of CD62P+ free platelets per µL of whole blood.

on α-CD41; region 2 contains PAg bound to leukocytes (LPAg) since they co-express fluorescence from FITC (α-CD41) and from PE on (α-CD45).

The relative abundance of PAg and LPAg changes with increasing agonist, as shown in Figure 4A for ADP and in Figure 4B for collagen. In both cases, a decline in PAg is accompanied by an increase in LPAg (expressed as a fraction of the total of all species). This is most evident at higher concentrations of agonists, where 60–80% of PAg are positive for WBC. These data imply that leukocytes are involved in forming the aggregated mass in platelet activation.

Figure 5 shows the strong correlation between increasing LPAg fraction and CD62P expression following ADP stimulation. This further confirms that exposure of P-selectin (CD62P) promotes interaction between platelets and WBC, and may explain why so little increase in CD62P+ is seen on free platelets (Fig. 2C). Specifically, Figure 6 shows the number of activated (CD62+) free platelets in whole blood compared to PRP, induced by ADP (1 µmol/L). It is seen that CD62P+ free platelets, following stimulation with ADP (1.0 mol/L), are more numerous in PRP than in whole blood; and, conversely, looking at the PAg, the number of CD62P+ is less in PRP than in whole blood at the same [ADP]. These observations are consistent with the assumption that in whole blood, expression of CD62P in platelets leads promptly to their binding to leukocytes (LPAg), depleting the supply of free CD62P+ platelets; but in PRP, where leukocytes are absent, the CD62P+ platelets can remain free.

Evaluation of PAg Assay in Certain Clinical Settings

Effect of desmopressin (DDAVP) on platelets. We recently reported on a direct effect of DDAVP on plate-

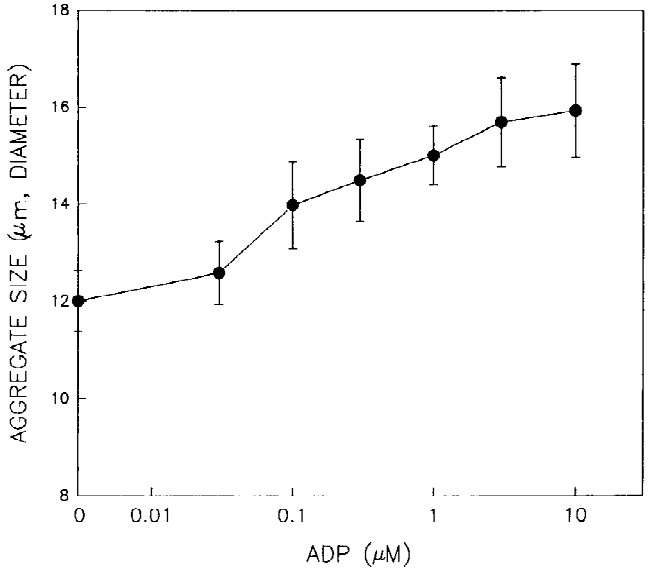

Fig. 3. Change of PAg size in relation to concentration of agonist [ADP]. Particle size was estimated by calibration of forward-scattered light with standard beads (5, 10, 15 μm diameter); bead diameter was a linear function of the log of forward scattered light (LFS). Since the PAg are not perfect spheres, the result shown does not necessarily reflect true absolute size.

lets, evidenced by increased shedding of PMP and internal calcium changes [17]. In the present study, DDAVP was added to whole blood and effects on PAg were examined, with results shown in Figure 7. DDAVP at nanomolar concentrations induced a modest but significant rise of CD62P+ PAg and rise in activated-free platelets. When studied by aggregometer (light transmittance), addition of DDAVP had no detectable effect at any concentration, in agreement with previous findings (referenced in [17]). We believe this is because the PAg produced by DDAVP are too small and/or few to be detected by transmittance aggregometry.

Effect of plasma from patients with thrombotic thrombocytopenic purpura (TTP). Observations by several workers have indicated that plasmas from at least some TTP patients may contain an aggregation promoting factor [18,19], but this was demonstrated by aggregometry in only a limited number of patients [18,19]. Figure 8B is a representative result following addition of TTP plasma to whole blood from a type O normal donor, to be compared with Figure 8A, the control receiving normal plasma (see legend). It is seen that TTP plasma promoted formation of activated (CD62P+) PAg, micro-PAg, and free platelets. This effect was seen in 13 of 14 (93%) of all TTP plasmas tested [16]; for details on controls, individual patients, and statistics, see figure 4 in [16]. When studied by lumi-aggregometry, none of the TTP plasmas had any detectable effect on aggregation or secretion. These findings demonstrate that flow cytometry is more sensitive to the pro-aggregatory effects of TTP plasma than aggregometry.

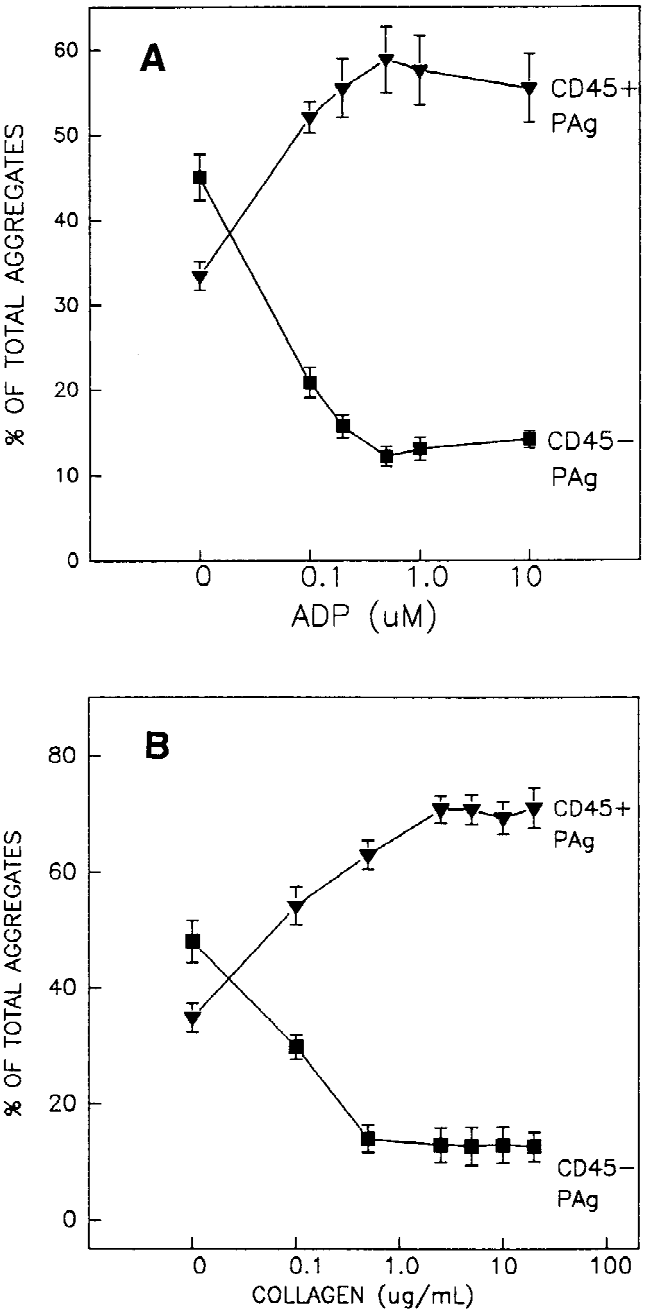

Fig. 4. Effects of agonist-induced activation on relative amounts of PAg and LPAg. The percent of total aggregates represented by PAg (■) and LPAg (▼) is shown as a function of increasing agonist concentration: ADP in A and collagen in B. The PAg are from region 4 of the histogram (see Fig. 1C,F) and the LPAg are from region 2 of the histogram.

DISCUSSION

A flow cytometric method for assaying PAg was described by Ault et al., who studied formation of PAg

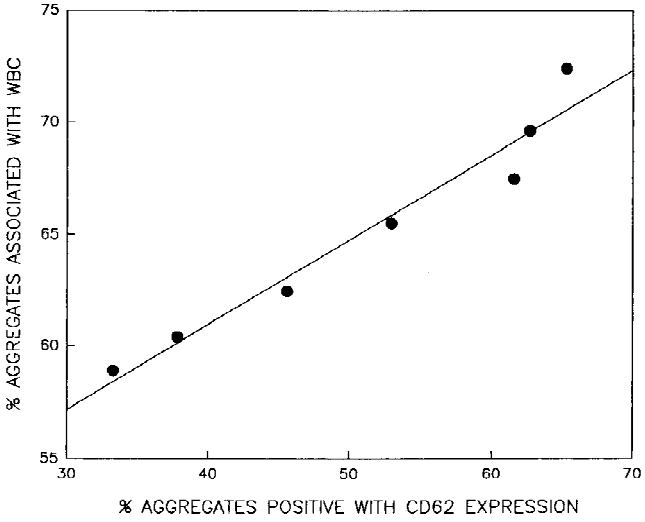

Fig. 5. Relationship between CD62P expression and formation of LPAg (leukocyte binding of platelets) in whole blood. The data points shown are from a representative experiment and were obtained by stimulation with ADP in the same range as Figure 2A (0–20 μmol/L). It is shown that the binding of platelets to leukocytes (forming LPAg) increases uniformly with increasing CD62P expression.

following incubation of whole blood (WBD) or PRP with agonists, and observed micro and macro platelet aggregates upon stimulation [7]. Galt et al. studied platelet aggregates in patients with peripheral vascular disease, but found no significant difference compared to healthy controls [8]. Rinder et al. studied platelet leukocyte interaction in WBD by a flow cytometric method and observed increased platelet/leukocyte aggregates (LPAg) in patients undergoing open heart surgery [20]. In the method of Rinder et al. [9], event recording was triggered by a pan-leukocyte marker (FITC labeled anti-CD45) and bound platelets were identified by co-expression of a platelet marker. Conversely, in the studies of Ault et al. [7] and Galt et al. [8], platelet aggregates were measured but not bound leukocytes. The method in this paper, initially reported in [15], combines quantitation of platelet aggregates (PAg), CD62P expression, and platelet-leukocyte interaction (LPAg) in a single whole-blood assay, and eliminates many of the time-consuming steps in the previously reported procedures [7–9].

The present flow cytometric method is 20- to 50-fold more sensitive than conventional aggregometry in detecting the early stages of platelet aggregation (incipient aggregation). This is shown by the much lower concentration of agonist needed to produce a measurable effect (Fig. 2). Because of the greater sensitivity of the flow cytometric method, DDAVP, which had no detectable effect by aggregometry, was shown to activate platelets in vitro (Fig. 7). Similarly, the effect of TTP plasma on normal platelets is readily detected by this flow cytometric technique [16] (Fig. 8) but only rarely can be detected by aggregometry [18]. These findings support the potential clinical value of this flow cytometric assay for platelet function studies.

In addition to increased sensitivity to agonists, the flow cytometric method provides information on platelet-leukocyte interaction (LPAg). The role of platelet-leukocyte interaction in thrombosis is receiving increasing attention in the recent literature. For example, the participation of neutrophils in reperfusion injury is well established [21–23], and a positive correlation between platelet-leukocyte interaction following cardiac bypass surgery was shown [20]. Activated platelets are capable of stimulating PMN [24]. Conversely, shear-induced platelet aggregability is potentiated by the presence of activated neutrophils, and these effects were said to be mediated by direct contact (adhesion between neutrophils and platelets) [25], or alternatively by neutrophil secretion of factors such as cathepsin G, a potent platelet activator [26]. Activated platelets adhere to monocytes and neutrophils (PMN) through specific ligands, and this adhesion was shown to be mediated mainly through the α-granular membrane protein P-selectin (CD62P; formerly GMP-140 or PADGEM) expressed on the platelet surface after activation [27–28], and Lewis × (CD15) or sialyl Lewis × molecules on the neutrophil or monocyte surface [29,30]. Our results further confirmed that the association of platelets with leukocytes was strongly correlated with CD62P expression in whole blood (Fig. 5). When samples of whole blood were compared to PRP by the flow cytometric method of this report, it was seen that about 10-fold more PAg were formed in whole blood than in PRP in response to the same level of agonist (Fig. 6). This suggests that leukocytes potentiate platelet activation and aggregation.

Elevated circulating PAg have been observed in patients with thrombotic disorders. In the early method for detecting PAg [31,32], blood was drawn into two tubes, one with EDTA (assumed to liberate free platelets from PAg) and the other with fixative (assumed to preserve PAg); the ratio of platelet counts in the two tubes was taken as an index of PAg in circulation. By this technique, elevated PAg were reported in patients with arterial insufficiency [32], hypoxemia [33], inflammatory bowel disease [34], and cerebral ischemia [35]. Increased PAg were also found in other disorders with a high risk of thrombosis. In recent studies, spontaneous constrast observed during cardiac ultrasound in some patients was found to be due to the formation of platelet aggregates in cardiac chambers; and these patients had a higher risk of thromboembolic complication [36]. Resolution of spontaneous contrast was reported with administration of the antiaggregating agent, trifluoperazine [37]. Flow cytometry appears preferable since it can quantitate numbers and sizes of PAg, activation status, and association with WBC simultaneously. As shown in Figure 4, higher con-

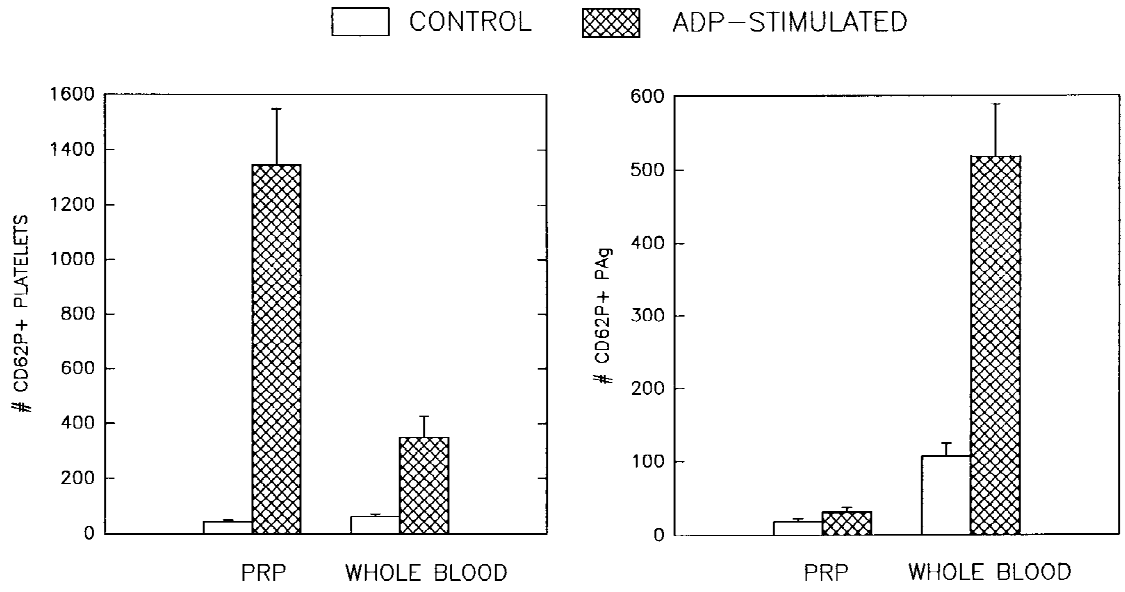

Fig. 6. Comparison of CD62P expressed in free platelets and platelet aggregates (PAg) as seen in whole blood vs. platelet-rich plasma (PRP). Values shown are means of four experiments, ± SD. Either whole blood or PRP received 1 µmol/L of ADP and was then assayed for PAg, LPAg, and free platelets as described in Methods.

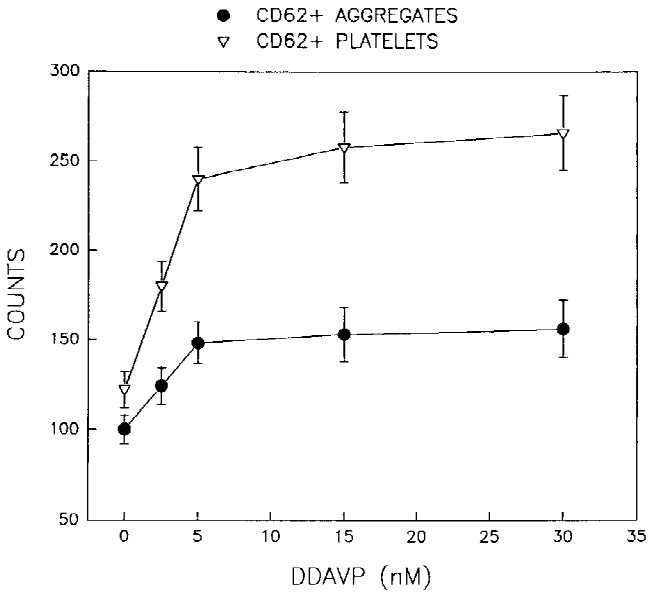

Fig. 7. Effect of desmopressin (DDAVP) on whole blood. Results show the mean of 6 experiments ± SD. The filled circles show CD62P+ PAg and the open triangles show CD62P+ free platelets. The 5 nmol/L level of DDAVP is approximately the level used in recommended therapeutic doses [17].

centrations of agonists induced higher percentages of leukocyte-platelet aggregates (LPAg) in a dose-dependent manner, indicating that LPAg formation is dependent on the degree of platelet activation. In vivo the role of LPAg in thrombosis remains mostly unknown. However, our preliminary results showed that LPAg were more dramatically increased (2- to 5-fold) than PAg (1.5- to 2-fold) in the thrombotic states of TTP [38] and heparin-induced thrombocytopenia with thrombosis (HITT) [39], showing that LPAg is more prominently associated with these thrombotic states than PAg.

The method described in this paper has been applied as an aid in monitoring the progress of TPP [16]: it was shown that CD62P+ PAg were elevated during active phases of the disease, were decreased following plasma infusion, and tended to normalize in remission. These changes were often seen prior to detection to changes in LDH or platelet count, the traditional indicators of TTP activity [16]. This method has also revealed platelet activation in Alzheimer's disease [40] and in multiple sclerosis [14].

The data presented here support the potential clinical utility of flow cytometric assay of these species (PAg, LPAg, CD62±) as indexes of platelet activation. It can detect subtle platelet abnormalities not detectable by conventional aggregometry and can enumerate PAg and LPAg.

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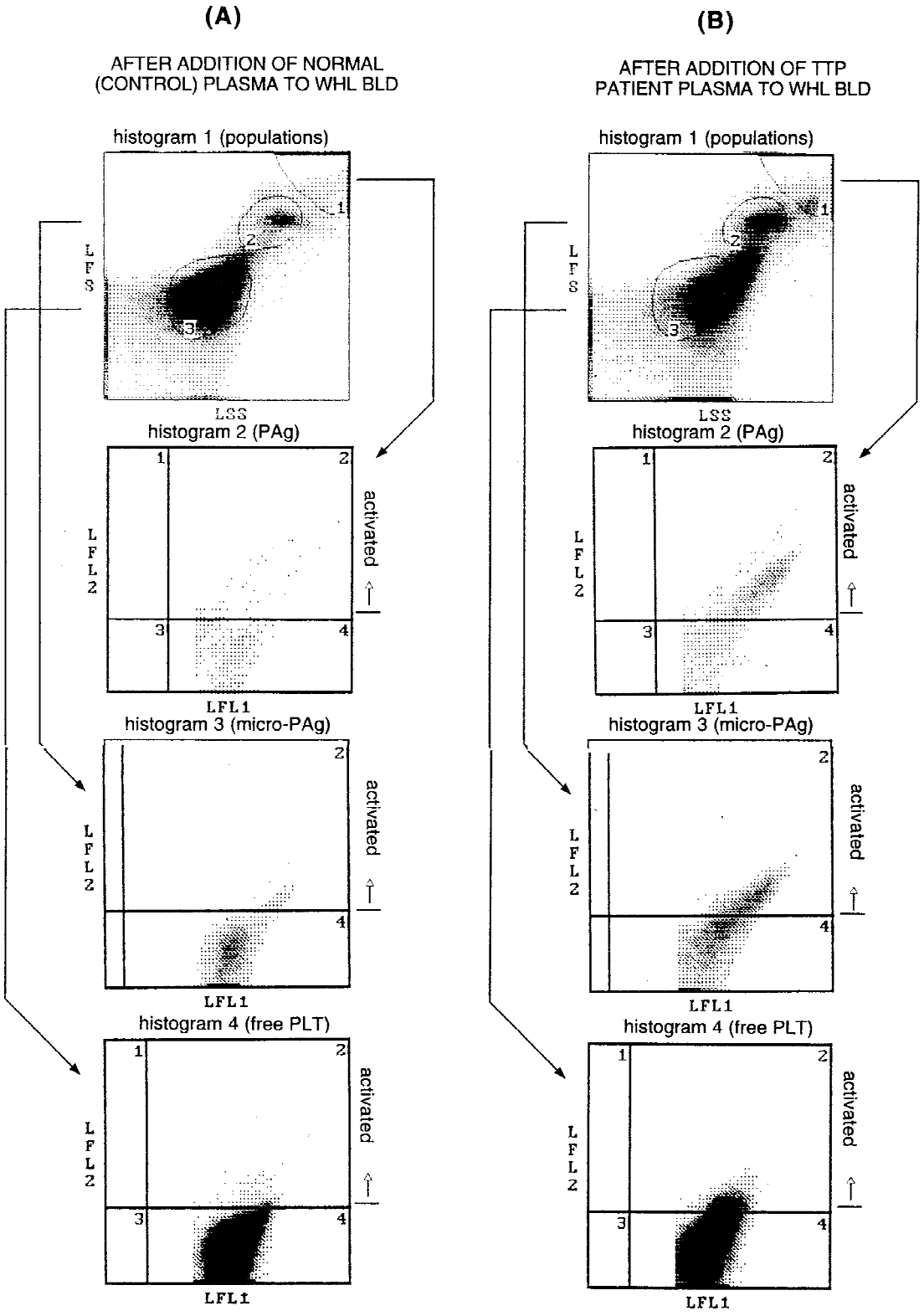

Fig. 8. Flow cytometric histograms illustrating the effect of addition of TTP plasma to normal whole blood. Either 100 µL normal plasma (A) or 100 µL plasma from a TTP patient (B), both stored frozen, was added to 50 µL of type O normal whole blood, not more than 2 h old, and was incubated with orbital shaking (120 rpm) at room temperature for 10 min, and prepared for flow cytometry as described in Methods. The top two hsitograms are the same as described for Figure 1A and D, and show the increased population of PAg and micro-PAg (bitmaps 1 and 2, respectively) following addition of TTP plasma. The lower histograms analyze the fluorescent signals from the populations in the bitmaps at top, as indicated by the arrows. LFL2 (log fluorescence of PMT 2) measures PE from CD62P expression; LFL 1 measures FITC from CD41 expression.

REFERENCES

1. Joseph M, ed: ''Immunopharmacology of Platelets.'' New York: Academic Press, 1995.
2. Colman RW, Cook JJ, Niewiarowski S: Mechanisms of platelet aggregation. In Coleman RW, Hirsh J, Marder VJ, Salzman EW (eds): ''Hemostasis and Thrombosis: Basic Principles and Clinical Practice,'' 3rd ed. Philadelphia: JB Lippincott Company, 1994, pp 508–524.
3. Siess W: Molecular mechanisms of platelet activation. Physiol Rev. 69:58–178, 1989.
4. Born GV: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 194:927–929, 1962.
5. Faint RW: Platelet-neutrophil interactions: Their significance. Blood Rev 6:83–91, 1992.
6. Dinerman JL, Mehta JL: Endothelial, platelet and leukocyte interactions in ischemic heart disease: Insights into potential mechanisms and their clinical relevance. J Am Coll Cardiol. 16:207–222, 1990.
7. Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS: Correlated measurement of platelet release and aggregation in whole blood. Cytometry. 10:448–455, 1989.
8. Galt SW, Mc Daniel MD, Ault KA, Mitchell J, Cronenwett JL: Flow cytometric assessment of platelet function in patients with peripheral arterial occlusive disease. J Vasc Surg. 14:747–756, 1991.
9. Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR: Activated and unactivated platelet adhesion to monocytes and neutrophils. Blood. 78:1760–1769, 1991.
10. Jy W, Horstman LL, Arce M, Ahn YS: Clinical significance of platelet microparticles in ITP. J Lab Clin Med 119:334, 1992.
11. Lee Y, Jy W, Horstman LL, Ahn YS. Elevated platelet microparticles in TIA, lacunar infarcts, and multiinfarct dementias. Thromb Res. 72:295, 1993.
12. Katopodis JN, Kolodny L, Jy W, Horstman LL, de Marchena EJ, Tao JG, Haynes DH, Ahn YS: Platelet microparticles and calcium homeostasis in acute coronary ischemias. Am J Hematol 54:95, 1997.
13. Ozner MD, Ahn YS, Horstman LL, Jy W, Myerburg RJ: Chronic platelet activation associated with acute coronary syndromes in 13 middle aged patients. Clin Appl Thromb/Hemost 3:46, 1997.
14. Kolodny L, Ahn YS, Sheremata WA: Evidence of platelet activation in multiple sclerosis. Presented at the 121st meeting of the Am Neurol Assoc, Miami, FL. Ann Neurol 40:520(Abstr M117), 1996.
15. Jy W, Horstman L, Park H, Mao W, Kolodny L, Ahn YS. Flowcytometric assay of platelet aggregates in whole blood: Detection of effects of TTP plasma and DDAVP. Blood 86(Suppl 1):899a, 1995.
16. Ahn YS, Jy W, Kolodny L, Mao WW, Valant P, Horstman LL: Activated platelet aggregates in thrombotic thrombocytopenic purpura: Decrease with plasma infusions and normalization in remission. Br J Haematol 95:408–415, 1996.
17. Horstman LL, Valle-Riestra BJ, Jy W, Wang F, Mao W, Ahn YS: Desmopressin (DDAVP) acts on platelets to generate microparticles and enhanced procoagulant activity. Thromb Res 79:163–174, 1995.
18. Lian EC-Y, Harkness DR, Byrnes JJ, Wallach H, Nunez R: Presence of platelet aggregating factor in the plasma of patients with TTP and its inhibition by normal plasma. Blood 53:333–338, 1979.
19. Lian, EC-Y, Siddiqui FA, Chen S-H, Feng L-M: Platelet-agglutinating/aggregating proteins from the plasma of patients with thrombotic thrombocytopenic purpura. In Kaplan BS, Trompeter RS, Moake JL (eds): ''Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura.'' New York: Marcel Dekker, Inc., 1992, pp 437–481.
20. Rinder CS, Bonan JL, Rinder HM, Mathew J, Hines R, Smith BR. Cardiopulmonary bypass induces leukocyte-platelet adhesion. Blood. 79:1201–1205, 1992.
21. Patarroyo M: Leukocyte adhesion in host defense and tissue injury. Clin Immunol Immunopathol. 60:333–348, 1991.
22. Verrier ED, Shen I: Potential role of neutrophil anti-adhesion therapy in myocardial stunning, myocardial infarction, and organ dysfunction after cardiopulmonary bypass. J Cardiac Surg. 8:309–312, 1993.
23. Von Andrian UH, Arfores KE. Neutrophil-endothelial cell interactions in vivo: A chain of events characterized by distinct molecular mechanisms. Agents Actions (Supp 1) 41:153–164, 1993.
24. Ruf A, Schlenk RF, Maras A, Morgenstern E, Patscheke H. Contact-induced neutrophil activation by platelets in human cell suspensions and whole blood. Blood. 80:1238–1246, 1992.
25. Rhee B-G, Hall ER, McIntire LV. Platelet modulation of polymorpho nuclear leukocytes shear induced aggregation. Blood. 67:240–246, 1986.
26. Molino M, Blanchard N, Belmonte E, Tarver AP, Abrams C, Hoxie JA, Cerletti C, Brass LF: Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. J Biol Chem. 270:11168–11175, 1995.
27. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B. PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell. 59:305–312, 1989.
28. Hamburger SA, McEver RP. GMP-140 mediates the adhesion of stimulated platelets to neutrophils. Blood. 75:550–554, 1990.
29. Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B. PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). Cell. 63:467–484, 1990.
30. Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S-I, Paulson JC. CD62 and endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-Lewis x. Proc Natl Acad Sci USA. 88:6224–6228, 1991.
31. Wu KK, Hoak JC: A new method for the quantitation and detection of platelet aggregation in patients with arterial insufficiency. Lancet II. 924–926, 1974.
32. Wu KK, Hoak JC: Spontaneous platelet aggregation in arterial insufficiency: Mechanism and implications. Thromb Haemost. 35:702–711, 1976.
33. Wedzicha JA, Syndercombe-Court D, Tan KC: Increased platelet aggregate formation in patients with chronic airflow obstruction and hypoxaemia. Thorax. 46:504–509, 1991.
34. Collins CE, Cahill MR, Newland AC, Rampton DS: Platelets circulate in an activated state in inflammatory bowel disease. Gastroenterology. 166:840–845, 1994.
35. Dougherty JH Jr, Levy DE, Weksler BB: Experimental cerebral ischemia produces platelet aggregates. Neurology. 29:1460–1465, 1979.
36. Daniel WG, Nellessen U, Schroder E, et al: Left atrial spontaneous echo contrast in mitral valve disease: An indicator for an increased thromboembolic risk. J Am Coll Cardiol. 11:1204–1211, 1988.
37. Mahony C, Sublett KL, Harrison MR: Resolution of spontaneous contrast with platelet disaggregatory therapy (trifluoperazine). Am J Cardiol. 63:1009–1010, 1989.
38. Valant P, Jy W, Horstman L, Mao W, Ahn YS: Increased leukocyte binding and P selectin expression in platelet aggregates induced by TTP plasma. Clin Res 44:327(A), 1996.
39. Valant P, Jy W, Horstman LL, Mao W, Kolodny L, Ahn YS: Leukocyte binding of platelet aggregates (PAg) in thrombotic thrombocytopenic purpura (TTP) and heparin induced thrombocytopenia with thrombosis (HITT). Presented at 4th Eur Symp Platelet Granulocyte Immunobiol, Hameenlinna, Finland, May 18–21, 1996.
40. Sevush S, Jy W, Horstman LL, Mallia RS, Mao WW, Ahn YS: Platelet activation in Alzheimer's disease. Arch Neurol 1997 (in press).